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Comparative studies of fungal degradation of single or mixed bioaccessible reactive azo dyes

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Abstract

A screening using several fungi (*Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes versicolor* and *Aureobasidium pullulans*) was performed on the degradation of syringol derivatives of azo dyes possessing either carboxylic or sulphonic groups, under optimized conditions previously established by us. *T. versicolor* showed the best biodegradation performance and its potential was confirmed by the degradation of differently substituted fungal bioaccessible dyes. Enzymatic assays (lignin peroxidase, manganese peroxidase, laccase, proteases and glyoxal oxidase) and GC-MS analysis were performed upon the assay obtained using the most degraded dye. The identification of hydroxylated metabolites allowed us to propose a possible metabolic pathway. Biodegradation assays using mixtures of these bioaccessible dyes were performed to evaluate the possibility of a fungal wastewater treatment for textile industries.

Keywords: White rot fungi; Textile dyes degradation; Ligninolytic enzymes; Wastewater treatment

1. Introduction

Azo dyes are released in large quantities into the environment from textile industries. These dyes are recalcitrant to microbial degradation, causing problems in the usual biological treatment of the industrial effluents (Swamy and Ramsay, 1999). Despite this, microbial degradation of azo dyes has been reported using different microorganisms (McMullan et al., 2001), bacteria (Rajaguru et al., 2000), yeasts (Martins et al., 1999) and filamentous fungi, such as the white rot fungi (Martins et al., 2001; Pointing, 2001). Due to the fungi oxidative mechanisms it is possible to avoid the formation of hazardous anilines, formed by reductive cleavage of the azo dyes, by other microorganisms such as bacteria (Chung and Stevens, 1993).

White rot fungi produce several enzymes that have been related to their ability to degrade natural polymers, such as lignin and cellulose, but can also degrade different synthetic chemicals, usually recalcitrant to biodegradation (Field et al., 1993; Knapp et al., 1995).

One of the well-characterized white rot fungi for industrial use is the basidiomycete *Phanerochaete chrysosporium*. The interest in this fungus is mainly due to the expression of some non-specific extracellular enzymes, as the ligninolytic peroxidases, that have been implicated in the degradation of dyes (Spadaro et al., 1992; Ollikka et al., 1993). The promising results obtained with this ligninolytic fungus, lead to the study of the potentialities of other species of ligninolytic basidiomycetes. According to this, biodegradation studies using *Trametes* and *Pleurotus* spp. have reported that production of laccase was highly related to lignin and

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dyes degradation (Platt et al., 1984; Thurston, 1994; Abadulla et al., 2000).

Although Basidiomycetes assume a noticeable importance in possible industrial application, other fungi, such as Deuteromycetes, have also been studied. *Aureobasidium pullulans* is an example of a deuteromycete with the ability to degrade industrial aromatic compounds such as the lignin breakdown products (Schoeman and Dickinson, 1997).

In this work and to our knowledge, it is the first time that the potential of several fungi (*P. chrysosporium*, *P. ostreatus*, *T. versicolor* and *A. pullulans*) on the biodegradation of fungal bioaccessible azo dyes (derivatives of syringol possessing either carboxylic or sulphonic groups) was tested and compared. *T. versicolor* showed the best results in this screening and its degradation potential was tested using eight fungal bioaccessible dyes synthesized by us (Martins et al., 2001). These results were compared to the biodegradation performance of *P. chrysosporium*, the most known ligninolytic fungus.

A mixture of the eight dyes, simulating an effluent, was also subjected to biodegradation assays with *P. chrysosporium* or *T. versicolor*. This constitutes an approach to a good strategy of environment protection, thinking of a fungal wastewater treatment for textile industries.

2. Materials and methods

2.1. Azo dyes

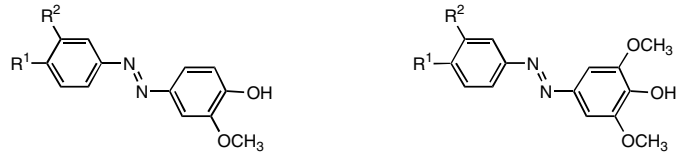
The eight dyes (Fig. 1) used in this work were synthesized using *meta* or *para* aminobenzoic or aminosulphonic acids as diazo components and two fungal bioaccessible groups present in lignin structure, guaiacol or syringol, as coupling components as described elsewhere (Martins et al., 2001).

2.2. Microorganisms

The strains, *P. chrysosporium* Burds (Burdsall and Eslyn) MUM 95.01, *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kummer MUM 94.08, *Trametes versicolor* (= *Coriolus versicolor*) Link Frenz (Quelef) MUM 94.04 and *A. pullulans* (De Bary) G. Arnaud MUM 94.10, used in this work were obtained from Micoteca da Universidade do Minho (MUM) culture collection. Stock cultures were maintained on TWAcellulose medium at 4 °C, with periodic transfer.

2.3. Media

The liquid culture medium, LCM (sucrose 5 $g\,l^{-1}$, ammonium sulphate 0.5 $g\,l^{-1}$, YNB—yeast nitrogen base without amino acids and ammonium sulphate 1.7 $g\,l^{-1}$, L-asparagine 1 $g\,l^{-1}$ and one of the dyes 50 $mg\,l^{-1}$) was used in the assays. The final pH was 4.5. The stock solutions of the dyes (100×), YNB (10×) and L-asparagine (10×) were filter-sterilized using 0.45 μm membranes. The same LCM, containing 50 $mg\,l^{-1}$ as total concentration of the mixture of the eight dyes, each one contributing with equal amounts of the stock solutions, was also performed in order to simulate a textile effluent. The pre-adaptation solid medium, PAM, with the same chemical composition of the LCM but containing only C*m*-s 50 $mg\,l^{-1}$ and agar 15 $g\,l^{-1}$ was also used, allowing the adaptation of the fungus to the dyes structure, as we



R^1 or R^2 = H unless stated

C*p*-g ($R^1 = CO_2H$): 4-aminobenzoic acid → guaiacol

C*p*-s ($R^1 = CO_2H$): 4-aminobenzoic acid → syringol

S*p*-g ($R^1 = SO_3H$): 4-aminobenzenesulphonic → guaiacol

S*p*-s ($R^1 = SO_3H$): 4-aminobenzenesulphonic → syringol

C*m*-g ($R^2 = CO_2H$): 3-aminobenzoic acid → guaiacol

C*m*-s ($R^2 = CO_2H$): 3-aminobenzoic acid → syringol

S*m*-g ($R^2 = SO_3H$): 3-aminobenzenesulphonic acid → guaiacol

S*m*-s ($R^2 = SO_3H$): 3-aminobenzenesulphonic acid → syringol

Fig. 1. Structure of the dyes. The nomenclature presented in this figure, expressing the diazo component → coupling component, is used in textile chemistry to suggest the synthesis process. The sigla used refer the kind of acid (carboxylic—C or sulphonic—S), its position relative to the azo bond (*meta*—*m* or *para*—*p*) and also the coupling component (guaiacol—g or syringol—s).

showed elsewhere (Martins et al., 2002). TWAcellulose, tap water agar (agar 15 $g\,l^{-1}$ in tap water) with a strip of cellulose paper, was used as an inducer medium of the enzymatic system of the studied fungi (Zacchi et al., 2000; Martins et al., 2001).

2.4. Culture conditions

An 8-mm diameter plug, cut with a sterile cork borer, from the periphery of a 7-day-old colony grown on a TWAcellulose culture plate, was inoculated at the centre of the PAM culture plate. Incubation was carried out for 7 days at 30 °C. For the single dye assays, 100 ml of LCM containing an azo dye in 250 ml Erlenmeyer flasks were inoculated with five plugs of 8 mm from the periphery of the PAM culture plate. For the decolourisation assays of an artificial textile effluent, 400 ml of LCM containing the eight azo dyes in 1000 ml Erlenmeyer flasks were inoculated with twenty plugs of 8 mm also from the periphery of the PAM culture plate. The liquid cultures were incubated at 30 °C in a Certomat rotary shaker at 150 rpm. Samples were collected after 1, 3, 5 and 7 days of incubation. Controls were carried out in the same conditions but without dyes or inoculum in order to compare, respectively, the variation of biomass and dye concentrations, with the results of the assays. The LCM inoculated and autoclaved was also used as control to study the possible contribution of adsorption dyes to fungal biomass.

Results presented are the means of a range of values obtained from the assays, and in the graphics, vertical error bars indicate the standard deviations from the average.

2.5. Dye, biomass and sucrose determinations

The concentration of dyes was determined by UV–visible spectroscopy by the decrease in the intensity of absorption at the maximum wavelength (λ_{max}) of the dye or group of dyes after samples scanning from 250 to 500 nm, using a spectrophotometer UV/VIS Jasco 7850. The absorbance value of the LCM medium containing the initial concentration of dye(s) corresponds to 100% of dye(s). The spectra were obtained from 1 ml of supernatant samples diluted with 2.5 ml of the appropriate buffer solution of citric acid/sodium hydrogenphosphate, pH = 4.4, as we previously tested (Martins et al., 2001).

Biomass concentration was determined by dry weight measurement after drying fungal cells at 105 °C for 24 h on a 0.45 μm pre-weighted membrane.

The sucrose concentration was determined colorimetrically, using the sucrose/D-glucose kit/test from Boehringer Mannheim (cat. no. 139041), reading the absorbance values at 340 nm. This kit was used as recommended by the manufacturer with the following modifications: (i) the blank sucrose sample was done with the sample solution but without the kit enzyme suspension; (ii) a miniaturized procedure was set up using a 300-μl microwell plate in an ELISA spectrophotometer SLT Spectra; and (iii) to obtain sucrose concentration a standard curve was constructed using the same sugar as standard. The initial sucrose concentration in LCM corresponds to 100%.

2.6. Enzymatic assays

All enzymatic assays were done at room temperature and determined colorimetrically. In the case of the LiP assay, a spectrophotometer UV/VIS Jasco 7850 was used and the activity of the others enzymes was determined using an ELISA spectrophotometer rainbow Tecan-sunrise, following a miniaturized procedure that was set up with a 300-μl microwell plate.

The lignin peroxidase, LiP (EC 1.11.1.14) assay was based on the oxidation of veratrylic alcohol by the increase in absorbance at 310 nm (Tien and Kirk, 1984). The reaction mixture contained: 1 ml of supernatant samples, 0.33 ml of 2 mM solution of veratrylic alcohol (3,4-dimethoxybenzylalcohol, Sigma-Aldrich) 0.33 ml of 0.15 $g\,l^{-1}$ 30% solution of hydrogen peroxide (Merck) and 1.34 ml of citric acid/sodium hydrogenphosphate buffer solution of pH 4.4.

The glyoxal oxidase, GLOX (EC 1.2.3.5) assay was based on the oxidation of methylglyoxal (Kersten and Kirk, 1987) by the increase in absorbance at 436 nm. The reaction mixture contained, in 300 μl: 10 μl of supernatant samples, 10 μl of a stock solution (100×) of 2.8 mM guaiacol (Sigma-Aldrich); 10 μl of 1 $mg\,l^{-1}$ solution of peroxidase (Merck), 50 μl of methylglyoxal (acetylformaldehyde, Sigma) and 120 μl of citric acid/sodium hydrogenphosphate buffer solution of pH 6.0.

The laccase, Lacc (EC 1.10.3.2) assay was based on the oxidation of syringaldazine (Givaudan et al., 1993) by the increase in absorbance at 525 nm. The reaction mixture contained, in 300 μl: 10 μl of supernatant samples, 90 μl of a 0.11 mM solution of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine, Sigma) in ethanol absolute (Merck) and 200 μl of citric acid/sodium hydrogenphosphate buffer solution of pH 6.0.

The Mn-dependent peroxidase, MnP (EC 1.11.1.13) assay was based on the oxidation of MBTH (Castillo et al., 1994) by the increase in absorbance at 590 nm. The reaction mixture contained, in 300 μl: 10 μl of supernatant samples, 10 μl of 0.23 $g\,l^{-1}$ 30% solution of hydrogen peroxide (Merck), 120 μl of 0.07 mM solution of MBTH (3-methyl-2-benzothiazoline hydrazone, Sigma), 10 μl of 0.3 mM solution of manganese (Merck) and 150 μl of citric acid/sodium hydrogenphosphate buffer solution of pH 4.4.

The proteases assay was based on the oxidation of azoprotein (Sarath et al., 1989) by the increase in

absorbance at 440 nm. The reaction mixture contained, in 300 μl: 10 μl of supernatant samples, 30 μl of 2% solution of azoprotein (sulfanilic-acid-azoalbumin, Sigma) in 50–100 mM buffer, 100 μl of a 10% solution of trichloroacetic acid (Riedel-de-Häen), 160 μl of citric acid/sodium hydrogenphosphate buffer solution of pH 6.0.

For each enzymatic activity assay, the same reaction mixture containing boiled supernatant samples instead of the supernatant ones was employed as a blank.

One unit (U) of enzyme activity was defined as the amount of the enzyme that caused a change in absorbance of 0.01 per minute under the assay conditions.

2.7. Extraction and derivatization procedures for GC-MS analysis

GC-MS analysis was performed on a biodegradation assay of C*m*-s at day 7, using 400 ml of LCM in a 1000 ml flask, in order to obtain a significant amount of organic extract. The supernatant, after filtration (0.45 μm), was extracted with ethyl acetate (3×150 ml). The organic extract was dried (Mg_2SO_4) and the solvent was removed under reduced pressure.

The organic residue (2 mg) was dissolved in pyridine 250 μl and the compounds containing hydroxyl and carboxyl groups were converted, respectively, into trimethylsilyl (TMS) ethers and esters, adding bis(trimethylsilyl)trifluoroacetamide 125 μl and trimethylchlorosilane 25 μl. After the mixture had stood at 70 °C for 30 min, the derivatized extract was analysed by GC-MS (Ekman, 1983).

2.8. GC-MS analysis

GC-MS analyses were performed using a Trace Gas Chromatograph 2000 Series with a Finnigan Trace MS mass spectrometer and equipped with a DB-1 J&W capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows: 35 cm s^{-1} as the carrier gas (He) flow rate; 80 °C as initial temperature for 5 min; 4 °C/min as temperature rate; 285 °C as final temperature for 10 min; 290 °C as injector and transfer-line temperature; 1:100 as split ratio. Compounds were identified, as TMS derivatives, by comparing their mass spectra with the GC-MS spectral library (Wiley-NIST).

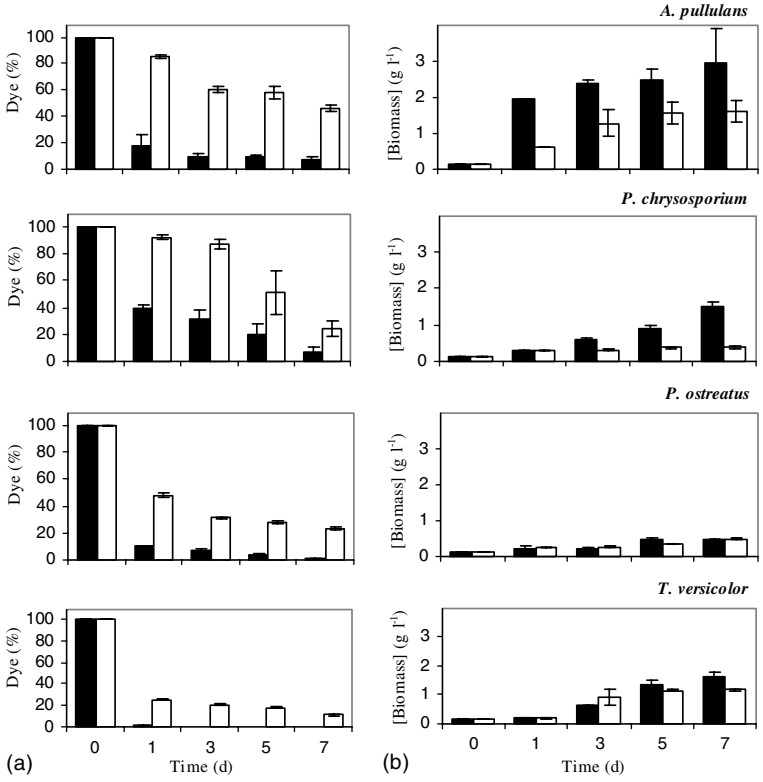


Fig. 2. Decolourisation of the LCM media containing C*m*-s (■) or S*m*-s (□) dyes (a) and the corresponding fungal biomass (b) of *A. pullulans*, *P. chrysosporium*, *P. ostreatus* and *T. versicolor*.

3. Results and discussion

In this work, the fungal screening on dye biodegradation was performed using either a carboxylic or a sulphonic azo dye, derivatives of syringol C*m*-s or S*m*-s (Martins et al., 2002). In these assays, a decrease in the intensity of absorption at the maximum wavelength (λ_{max}) of the dye, during the 7 days of incubation, was shown. For all studied fungi, the disappearance of sucrose of the LCM occurred, being already less than 10% in the first day of assay. Concomitantly the decrease of dye concentration and the increase of the biomass concentration occurred (Fig. 2) with no significative adsorption of dyes to the fungal biomass, which was confirmed using a heat-killed control.

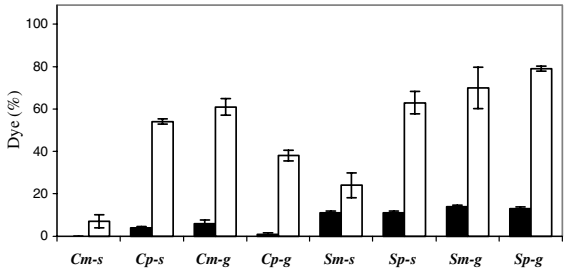


Fig. 3. Decolourisation of LCM containing each of the dyes, by *T. versicolor* (■) or *P. chrysosporium* (□), after 7 days of incubation.

The higher percentages of decolourisation, 76–100%, of the LCM containing S*m*-s or C*m*-s, were obtained with the white rot fungi *T. versicolor* followed by *P. ostreatus* (Fig. 2a). The higher values of biomass were observed in the assays using *A. pullulans* showing the less extent of decolourisation of the LCM containing S*m*-s (Fig. 2).

Some differences in the enzymatic system of the fungi used in this work can explain the different patterns of decolourisation and biomass increase observed. The higher percentages of decrease of dyes concentration obtained with *T. versicolor* and *P. ostreatus* can be explained by the presence of laccase in the LCM since, this extracellular enzyme is not produced by the other strains studied (Call and Mücke, 1997; Martins et al., 2001; Podgornik et al., 2001). *T. versicolor* was selected as the fungus with the best performance of dye biodegradation (Fig. 2) and we proceed with the determination of enzymatic activities in the C*m*-s biodegradation assay. These assays allowed the detection of Lacc activity 1.4 U ± 0.0 at day 1, 2.0 U ± 0.1 at day 3, 2.1 U ± 0.2 at day 5 and 1.4 U ± 0.1 at day 7, lower values of LiP activity 0.4 ± 0.1 at day 3 and 0.2 ± 0.1 at day 7 and GLOX residual activity. MnP and proteases were never detected.

Since the best results were obtained using *T. versicolor*, the decolourisation of the LCM containing each of the eight dyes using this species and *P. chrysosporium*, the most studied white rot fungus, was compared

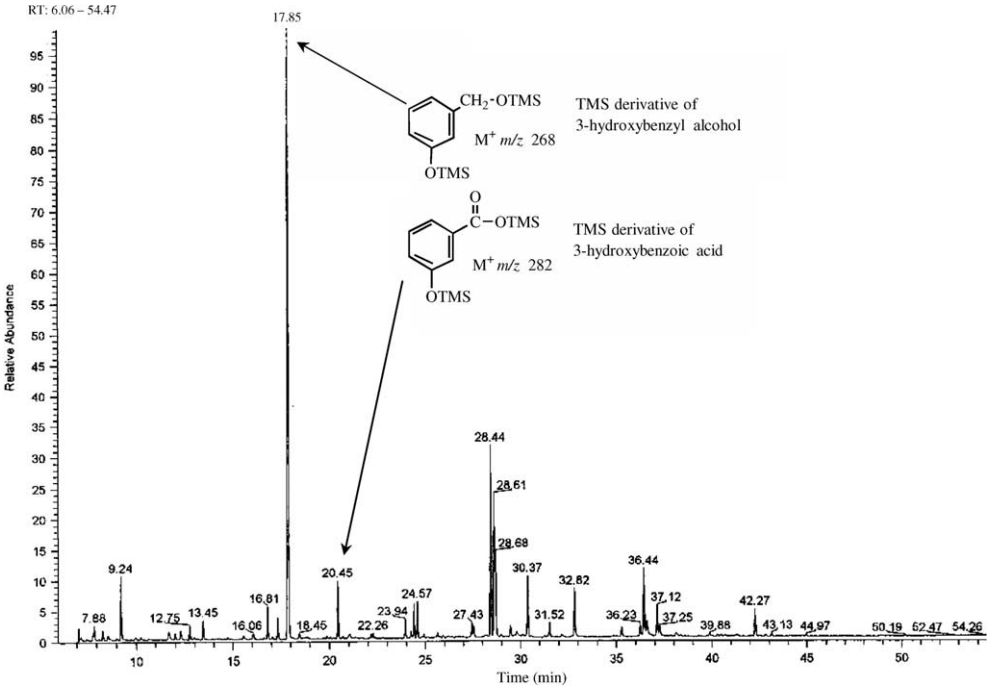


Fig. 4. Chromatogram of GC-MS analysis of C*m*-s degradation by *T. versicolor*, after 7 days of incubation.

(Fig. 3). *T. versicolor* was able to decolourise all the LCM more efficiently than *P. chrysosporium*, the carboxylic dyes being better degraded than the sulphonic.

GC-MS analysis of a day 7 sample extract of C*m*-s degradation by *T. versicolor*, after TMS derivatization, allowed the identification of key metabolites resulting from aromatic ring hydroxylation after cleavage of the azo bond (N=N). In Fig. 4 the peak having a retention time of 20.45 min corresponds to *meta*-hydroxybenzoic acid-TMS derivative (molecular ion—M^+ m/z 282) and the peak having a retention time of 17.85 min corresponds to *meta*-benzyl alcohol-TMS derivative (M^+ m/z 268). Taking into account that these hydroxylated metabolites are precursors of catechol derivatives, which are essential to aromatic ring cleavage, as claimed by other authors (Hofrichter and Scheibner, 1993), it is possible to propose a metabolic pathway towards central metabolism (Fig. 5).

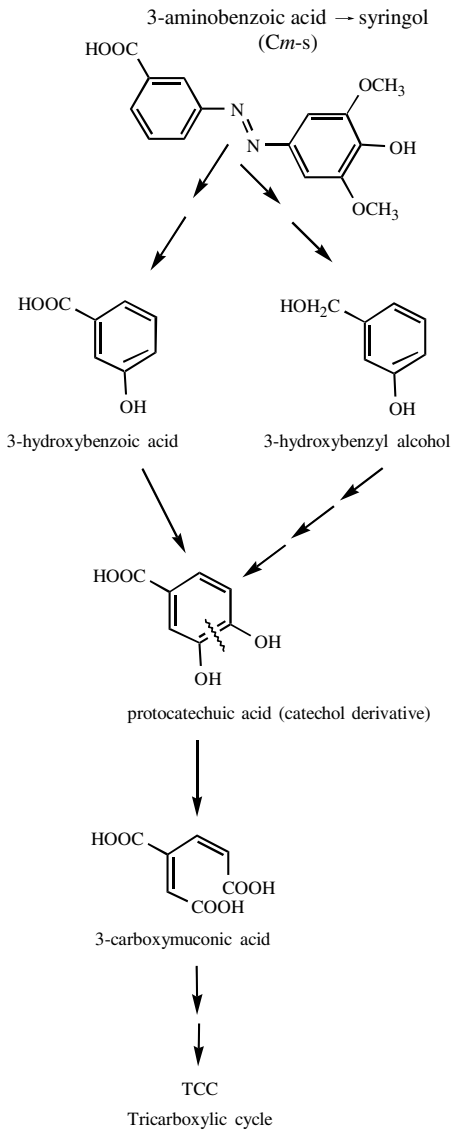


Fig. 5. Proposed metabolic pathway from the identified C*m*-s metabolites towards mineralisation, using *T. versicolor*.

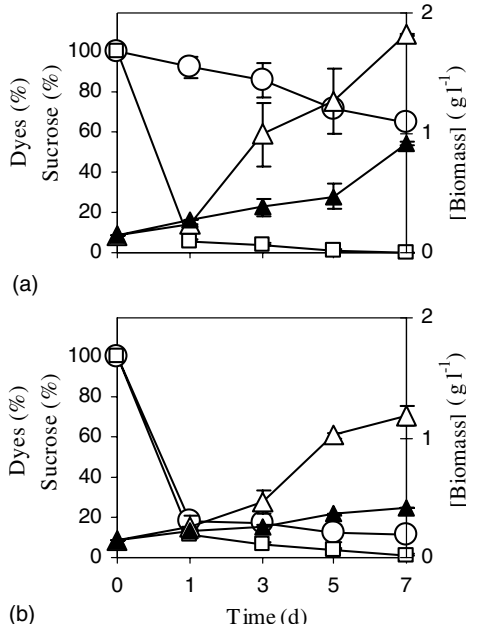


Fig. 6. Time courses of dyes (○), sucrose (□), biomass (△) and control biomass (▲) concentrations determined during the decolourisation of the simulated textile effluent by *P. chrysosporium* (a) or *T. versicolor* (b).

From the results obtained it was decided to mix the eight dyes simulating a textile effluent and to perform biodegradation assays with *P. chrysosporium* or *T. versicolor*. Considering the results obtained with the LCM containing the single dyes (Fig. 2), a similar decolourisation profile of the simulated effluent was obtained since *T. versicolor* showed, earlier than *P. chrysosporium*, higher percentages of decolourisation (Fig. 6).

The decolourisation observed in the LCM containing the single or the mixture of dyes points to dyes biodegradation since, without dye adsorption, the biomass increases along with the decrease of dyes concentration even after the disappearance of sucrose of the LCM, showing higher concentration values than the biomass-control without dyes (Fig. 6).

4. Conclusions

T. versicolor proved to be the most suitable fungus for the degradation of the dyes studied, either single or mixed, presenting the highest percentages of decolourisation with the lowest values of biomass. The identification of two hydroxylated metabolites from the

degradation of the most degraded dye, allowed the proposition of a metabolic pathway. Using these fungal bioaccessible azo dyes and due to the oxidative fungal mechanisms, no hazardous anilines, resulting from reductive cleavage of the azo bond were detected. The results obtained point to the advantages of the possible use of this "green" bioaccessible dyes in the textile industry, thinking of a biological effluent treatment using ligninolytic fungi.

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References

Abadulla, E., Tzanov, T., Costa, S., Robra, K.-H., Cavaco-Paulo, A., Gübitz, G.M., 2000. Decolourisation and detoxification of textile dyes with laccase from *Trametes hirsuta*. Appl. Environ. Microbiol. 66, 3357–3362.

Call, H.P., Mücke, I., 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). J. Biotechnol. 53, 163–202.

Castillo, M.P., Stenström, J., Ander, P., 1994. Determination of manganese peroxidase activity with 3-methyl-2-benzothiazoline hydrazone and 3-(dimethylamino)benzoic acid. Anal. Biochem. 218, 399–404.

Chung, K.-T., Stevens Jr., B., 1993. Degradation of azo dyes by environmental microorganisms and helminthes. Environ. Toxicol. Chem. 12, 2121–2132.

Ekman, R., 1983. Suberin monomers and triterpenoids from the outer bark of *Betula verrucosa*, Ehrh. Holzforschung 37, 205–211.

Field, J.A., De Jong, E., Feijoo-Costa, G., De Bont, J.A.M., 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. 11, 44–49.

Givaudan, A., Effose, A., Faure, D., Portier, P., Bouillant, M.-L., Bally, R., 1993. Phenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. FEMS Microbiol. Lett. 108, 205–210.

Hofrichter, M., Scheibner, K., 1993. Utilization of aromatic compounds by the *Penicillium* strain Bi 7/2. J. Basic Microbiol. 33, 227–232.

Kersten, P.J., Kirk, T.K., 1987. Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J. Bacteriol. 169, 2195–2201.

Knapp, J.S., Newby, P.S., Reece, L.P., 1995. Decolorization of dyes by wood-rotting basidiomycete fungi. Enzyme Microb. Technol. 17, 664–669.

Martins, M.A.M., Cardoso, M.H., Queiroz, M.J., Ramalho, M.T., Oliveira-Campos, A.M., 1999. Biodegradation of azo dyes by the yeast *Candida zeylanoides* in batch aerated cultures. Chemosphere 38, 2455–2460.

Martins, M.A.M., Ferreira, I.C., Santos, I.F.M., Queiroz, M.J., Lima, N., 2001. Biodegradation of bioaccessible textile azo dyes by *Phanerochaete chrysosporium*. J. Biotechnol. 89, 91–98.

Martins, M.A.M., Queiroz, M.J., Silvestre, A.J.D., Lima, N., 2002. Relationship of chemical structures of textile dyes on the pre-adaptation medium and the potentialities of their biodegradation by *Phanerochaete chrysosporium*. Res. Microbiol. 153, 361–368.

McMullan, G., Meehan, C., Conneely, A., Kirby, N., Robinson, T., Nigam, P., Banat, I.M., Marchant, R., Smyth, W.F., 2001. Microbial decolourisation and degradation of textile dyes. Appl. Microbiol. Biotechnol. 56, 81–87.

Ollikka, P., Alhonmaki, K., Leppanen, V.-L., Glumoff, I.R., Suominnen, I., 1993. Decolorization of azo, triphenyl methane heterocyclic, and polymeric dyes by lignin peroxidase isoenzyme from *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 59, 4010–4016.

Podgornik, H., Stegu, M., Zibert, E., Perdih, A., 2001. Laccase production by *Phanerochaete chrysosporium*—an artefact caused by Mn(III)? Lett. Appl. Microbiol. 32, 407–411.

Platt, M.W., Hadar, Y., Chet, I., 1984. Fungal activities involved in lignocellulose degradation by *Pleurotus*. Appl. Environ. Microbiol. 20, 150–154.

Pointing, S.B., 2001. Feasibility of bioremediation by white-rot fungi. Appl. Microbiol. Biotechnol. 57, 20–33.

Rajaguru, P., Kalaiselvi, K., Palanivel, M., Subburam, V., 2000. Biodegradation of azo dyes in a sequential anaerobic-aerobic system. Appl. Microbiol. Biotechnol. 54, 268–273.

Sarath, G., Motte, R.S., Wagner, F.W., 1989. Protease assay methods. In: Beynon, R.J., Bond, J.S. (Eds.), Proteolytic Enzymes—A Practical Approach. Oxford University Press, Oxford, England, pp. 25–30.

Schoeman, M., Dickinson, D., 1997. Growth of *Aureobasidium pullulans* on lignin breakdown products at weathered wood surfaces. Mycologyst 11, 168–172.

Spadaro, J.T., Gold, M.H., Renganathan, V., 1992. Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58, 2397–2401.

Swamy, J., Ramsay, J.A., 1999. The evaluation of white rot fungi in the decolouration of textile dyes. Enzyme Microb. Technol. 24, 130–137.

Thurston, C.F., 1994. The structure and function of fungal laccases. Microbiology 140, 19–26.

Tien, M., Kirk, T.K., 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization and catalytic properties of a unique H_2O_2 requiring oxygenase. Proc. Natl. Acad. Sci. USA 81, 2280–2284.

Zacchi, L., Burla, G., Zuolong, D., Harvey, P.J., 2000. Metabolism of cellulose by *Phanerochaete chrysosporium* in continuously agitated culture is associated with enhanced production of lignin peroxidase. J. Biotechnol. 78, 185–192.